A Potent, Nonpeptidyl 1*H*-Quinolone Antagonist for the Gonadotropin-Releasing Hormone Receptor

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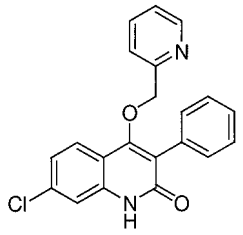
Extensive development of the structure−activity relationships of a screening lead determined three important pharmacophores for gonadotropin-releasing hormone (GnRH) receptor antagonist activity. Incorporation of the 3,4,5-trimethylphenyl group at the 3-position, 2-(2(*S*)-azetidinyl)ethoxy group at the 4-position, and *N*-4-pyrimidinylcarboxamide at the 6-position of the quinolone core resulted in the identification of 4-(2-(azetidin-2(*S*)-yl)ethoxy)-7-chloro-2-oxo-3-(3,4,5-trimethylphenyl)-1,2-dihydroquinoline-6-carboxylic acid pyrimidin-4-ylamide (**1**) as a potent antagonist of the GnRH receptor. A 10^4-fold increase in in vitro binding affinity is observed for the GnRH receptor as compared to the initial screening lead. Compound **1** exhibits nanomolar binding activity and functional antagonism at the human receptor and is 7-fold less active at the rhesus receptor. Intravenous administration of compound **1** to rhesus monkeys results in a significant decrease of the serum levels of downstream hormones, luteinizing hormone (79% decrease in area under the curve) and testosterone (92% decrease in area under the curve), at a dose of 3 mg/kg. Quinolone **1** is a potent nonpeptidyl antagonist for the human GnRH receptor that is efficacious for the suppression of luteinizing hormone and testosterone in primates.

Introduction

Gonadotropin-releasing hormone (GnRH) is a decapeptide released by the hypothalamus which binds to receptors on the pituitary.[1] The activation of this G-protein coupled receptor[2] causes the release of luteinizing hormone and follicle-stimulating hormone which regulate gonadal steroid hormone production. A variety of disease conditions such as prostate cancer, breast cancer, and endometriosis may be treated by suppression of the hypothalamic−pituitary−gonadal hormonal axis. There is recent clinical evidence that peptidic GnRH antagonists directly lower sex hormone levels alleviating disease symptoms with a superior side effect profile as compared to other therapies.[3] Therefore, the development of small molecule antagonists of the GnRH receptor may be clinically useful without the usual liabilities associated with large peptidyl therapeutics.[4] In this paper, we report the identification of a nonpeptidyl 3-arylquinolone antagonist of the GnRH receptor including in vivo efficacy in a primate model for hormone suppression.

Earlier publications from this laboratory disclosed the initial screening efforts and the identification of a 4-alkoxy-7-chloroquinolone lead structure (Figure 1).[5] Extensive development of the structure−activity relationships (SARs) of our lead identified three important pharmacophores for GnRH receptor antagonist activity. Incorporation of the 3,4,5-trimethylphenyl group[6] at the 3-position, 2-(2(*S*)-azetidinyl)ethoxy group[7] at the 4-position, and *N*-4-pyrimidinylcarboxamide[8] at the 6-position of the quinolone core resulted in the identification of compound **1** as a potent antagonist of the human GnRH receptor.



Merck Lead

Figure 1. Merck lead compound.

Results and Discussion

Chemistry. The synthetic route to quinolone **1** is similar to those reported in the earlier publications from our group (Scheme 1).[5−8] Treatment of methyl 4-chloroanthranilate (**2**) with iodine and silver sulfate provided a quantitative crude yield of the 5-iodinated derivative. Acetylation of the amino group with acetyl

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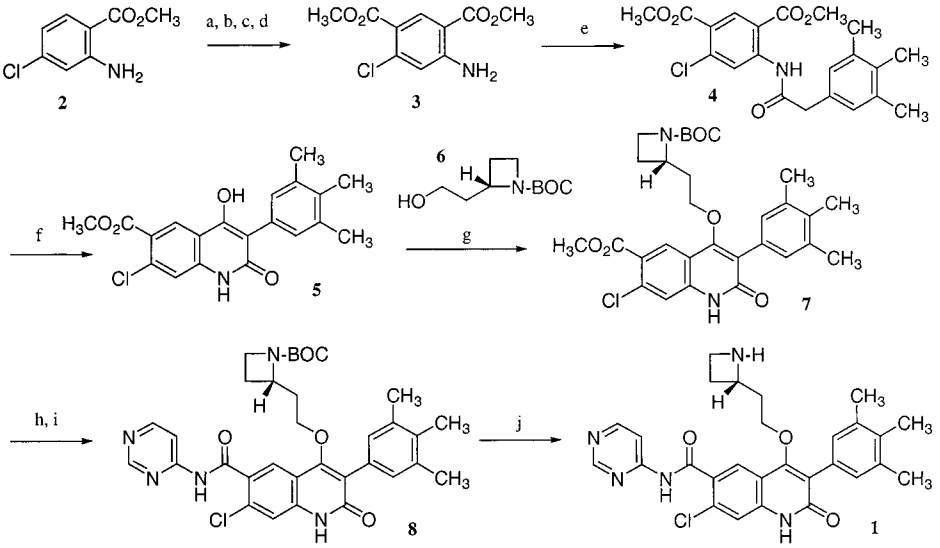
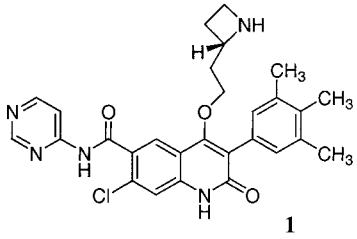
Scheme 1. Synthesis of Compound **1**[a]



[a] Reagents and conditions: (a) I_2, Ag_2SO_4, EtOH, rt, 1 h, 100% crude yield; (b) AcCl, 1,2-dichloroethane, 80 °C, 3 h, 93%; (c) $(PPh_3)_2PdCl_2$, CO (1 atm), Et_3N, DMF/MeOH (4:1), 95 °C, 16 h, 74%; (d) H_2SO_4, MeOH, reflux, 1 h, 80%; (e) 3,4,5-trimethylphenylacetyl chloride, 1,2-dichloroethane, 80 °C, 3 h, 78%; (f) $LiN(TMS)_2$, THF, 0 °C, 2 h, 82%; (g) alcohol **6**, PPh_3, DEAD, THF, 0 °C to rt, 24 h; (h) LiOH, $THF/H_2O/EtOH$, 80 °C, 5 h, 86%, 2 steps; (i) 4-aminopyrimidine, EDAC, DMAP, CH_2Cl_2, rt, 16 h, 84%; (j) $CF_3CO_2H-CH_2Cl_2$ (1:1), rt, 3 h, 87%.



Compound 1 *in vitro* data[a]

Species	Binding IC_{50} (nM)	PI Turnover IC_{50} (nM)
human	0.44 ± 0.39	1.0 ± 0.6
rhesus	0.5	7.0 ± 2.2
rat	4.0	81
dog	60.0	350

[a] in vitro data represents an average of two experiments (n = 2); standard error given in cases where n ≥ 4.

Figure 2. In vitro characterization of compound **1**.

chloride was necessary to prevent side reactions in the ensuing carbonylation. Reaction of the aryl iodide with a Pd-catalyst under carbon monoxide atmosphere in *N*,*N*-dimethylformamide/methanol solution provided the isophthalate diester. Filtration through a silica gel pad to remove the palladium byproducts followed by acetamide hydrolysis in sulfuric acid/methanol afforded the crystalline diester **3**.

Acylation of intermediate **3** with the acid chloride of 3,4,5-trimethylphenylacetic acid[9] gave the crystalline amide **4** in 78% yield. Quinolone formation under basic conditions followed by aqueous acid quench, filtration, and trituration with cold acetonitrile provided the 4-hydroxyquinolone **5** in 82% yield. This key intermediate was dried in vacuo to remove any residual water which would interfere with the subsequent coupling reaction. The protected (*S*)-azetidineethanol derivative **6** was prepared in several steps from (*S*)-azetidine-2-carboxylic acid with the key transformation being the Arndt−Eistert homologation.[10] Coupling of the two fragments **5** and **6** was accomplished by utilizing our previously reported Mitsunobu protocol.[7] Silica gel chromatography of the crude reaction mixture provided the desired 4-alkylated quinolone **7** contaminated with a minor amount of diethyl *N*,*N*′-hydrazinedicarboxylate byproduct which is removed in the next step. Hydrolysis of the methyl ester under basic conditions, followed by acidic workup, afforded the 6-carboxylic acid intermediate free of any impurities in 86% yield for the two steps. Amide formation under anhydrous conditions with 4-aminopyrimidine gave the desired amide **8** in 84% yield. Removal of the BOC group with trifluoroacetic acid provided compound **1** in 87% yield.

In Vitro Characterization. The initial SARs of the screening lead were developed based on data from a rat pituitary membrane binding assay.[5] Later, a radioligand binding assay using cloned CHO cells expressing the human GnRH receptor[6−8] supplemented the data provided by the initial rat pituitary membrane radioligand binding assay. In addition, an assay to determine functional antagonism of GnRH-stimulated phosphatidyl inositol (PI) hydrolysis in CHO cells expressing cloned human GnRH receptors was developed (PI turnover assay). All in vitro data are expressed as the IC_{50} for inhibition of binding (vs [^{125}I]buserelin) or functional antagonism (vs GnRH) at the GnRH receptor of the particular species for which the assay is being conducted.

Compound **1** possesses an $IC_{50} = 0.44 \pm 0.39$ nM in the cloned human GnRH receptor membrane binding assay and excellent functional antagonism at the human GnRH receptor, $IC_{50} = 1.0 \pm 0.6$ nM (Figure 2). A significant decrease in binding and functional activity is observed for the rat and dog receptors (10- and 150-fold shifts in binding, respectively) despite the high degree of receptor identity between the four species.[11]

Compound **1** shows equal binding affinity to the rhesus GnRH receptor (IC_{50} = 0.5 nM) and only a 7-fold shift in functional antagonist activity (7.0 ± 2.2 nM) as compared to the human receptor. This result dictated that any proof of concept study in animal models be performed in primates such as rhesus monkey.

In Vivo Studies with Compound 1. Compound **1** was studied in rhesus macaques to determine the efficacy for hormone suppression in vivo. Effective antagonism of the GnRH receptor would be expected to block production of the downstream hormones luteinizing hormone (LH) and testosterone (T). Earlier attempts to explore efficacy of quinolone **1** in a rat LH-lowering model were only moderately successful due to the 100-fold decrease in intrinsic potency in vitro relative to the human receptor along with extremely high plasma clearance of the compound. A more informative study was performed in rhesus monkeys, a species in which compound **1** has only a 7-fold shift in functional antagonism in vitro.

Rhesus iv pharmacokinetics were determined in a separate study to ensure proper drug exposure prior to utilization of this species as an animal model. Compound **1** gave, after a 0.5 mg/kg iv dose, a plasma drug level area under the curve (AUC) of 303 ± 11 ng·h/mL, a plasma clearance (Cl_p) of 30 ± 0.7 mL/min/kg, and a terminal half-life ($t_{1/2}$) of 9.5 h. Similarly at the higher 3 mg/kg dose, AUC of 1324 ± 134 ng·h/mL, Cl_p of 33 ± 3.4 mL/min/kg, and $t_{1/2}$ of 5 ± 0.7 h were observed. These results confirmed that adequate exposure of compound **1** could be obtained in the rhesus macaques to warrant further study.

Rhesus macaques were dosed iv with vehicle (ethanol/PEG400/saline, 10/40/50) or compound **1** (in the same vehicle) at doses of 0.5 and 3 mg/kg, and serum LH and T levels were determined by RIA from blood samples taken at 20-min intervals. In these experiments, each monkey served as its own control due to the variability and pulsatile nature of the blood levels of both hormones, with vehicle and each drug dose administration occurring on separate days. Previously conditioned rhesus monkeys were chronically catheterized in order to allow dosing and blood sampling without disturbing the animals. The animals were fed on a daily schedule with a coordinated light−dark cycle. Sampling occurred for 130 min prior to dosing of the vehicle or antagonist to establish basal levels and to determine that the sampling system was functioning properly. Dark cycle for these animals began 30−50 min after the administration of the compound, since in male rhesus there is a diurnal rhythm of LH and T, with highest levels observed at night. The time course data for one representative animal are presented below. (See Supporting Information for data for all monkeys included in this study.)

Figure 3 shows LH levels after vehicle dosing and demonstrates the pulsatile nature of serum LH levels with a large increase in LH after the beginning of the dark cycle (t > 50 min). Intravenous administration of the 0.5 mg/kg dose of compound **1** resulted in a significant decrease in the amplitude of the LH peaks. Exposure to compound **1** at a higher 3 mg/kg iv dose results in complete suppression of LH peaks for the duration of the study.

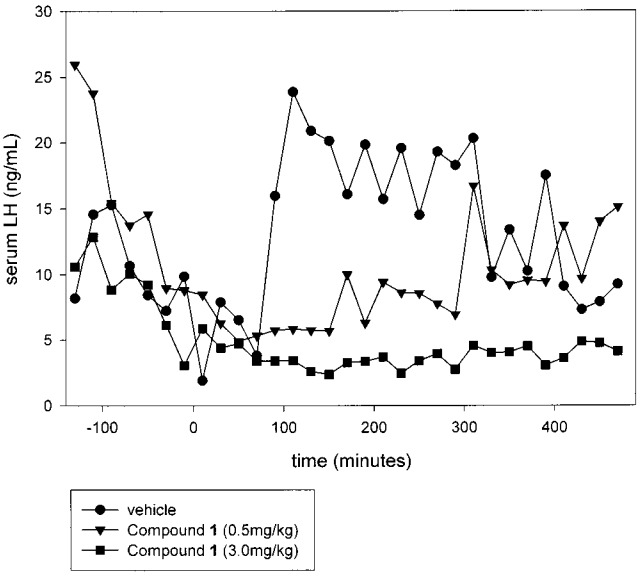


Figure 3. Rhesus LH levels.

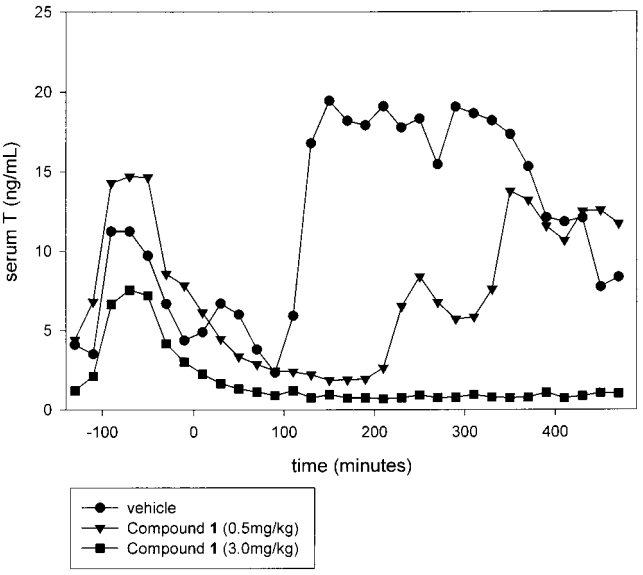


Figure 4. Rhesus T levels.

Serum T levels were also determined from the blood samples removed at each time point (Figure 4). A significant decrease in serum T was observed at the lower dose, but rebound to control levels was observed at 6 h postdose. At the higher dose, suppression of T to near castrate levels was observed for the duration of the study.

The time course data for the four monkeys in the study were combined to calculate hormone AUC for LH and T levels for the duration of the experiment (t = −130 to 470 min). The average calculated AUC for LH in the vehicle arm of the study was determined to be 320 ± 96 ng·h/mL (Figure 5). In comparison, LH AUC was reduced by an average of 41% (176 ± 64 ng·h/mL, p = 0.02) at the 0.5 mg/kg dose and 79% (62 ± 32 ng·h/mL, p = 0.0002) at the 3 mg/kg dose, respectively. Mean T AUC for the vehicle-treated leg of the study was 325 ± 118 ng·h/mL with an average reduction of 54% (145 ± 21 ng·h/mL, p = 0.006) at the lower dose and a 92% reduction (28 ± 25 ng·h/mL, p = 0.0004) in T AUC at the higher dose of compound **1** (Figure 5).

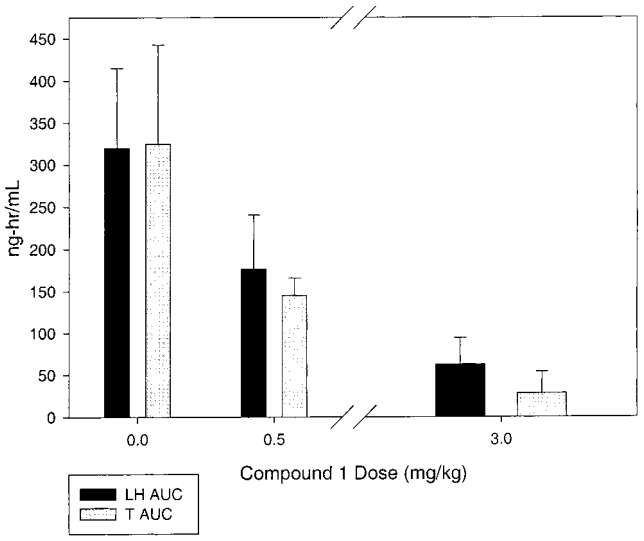


Figure 5. Effect of compound **1** on hormone AUC in rhesus macaques.

Conclusion

We have reported the design and synthesis of compound **1** as a potent antagonist of the human GnRH receptor. Significant improvements in antagonist activity for this lead class were achieved by incorporation of a 3,4,5-trimethylphenyl group at the 3-position, 2-((*S*)-2-azetidinyl)ethoxy group at the 4-position, and *N*-(4-pyrimidinyl)carboxamide at the 6-position of the quinolone core contained in the initial lead structure. These modifications resulted in a 10^4-fold improvement of the binding and functional activity in vitro. Compound **1** exhibits nanomolar functional antagonism at the human GnRH receptor and is 7-fold less active at the rhesus receptor. Intravenous administration of compound **1** to rhesus macaque at a dose of 3 mg/kg resulted in a significant decrease in the blood levels of downstream hormones: LH (79% decrease in AUC) and T (92% decrease in AUC). Quinolone **1** is a potent nonpeptidyl antagonist of the human GnRH receptor that is efficacious for the suppression of LH and T in primates.

Experimental Section

Chemistry. General Methods. ^{1}H NMR spectra were recorded on Varian XL series spectrometers at the indicated field strengths. Low-resolution mass spectral analyses were obtained with a LKB 9000 at an ionizing voltage of 70 eV. High-resolution mass spectral analysis was obtained using a Finnigan New Star FT/ICR with electrospray ionization. Reagents, solvents and drying agents were obtained from commercial sources and used without further purification or drying with the exception of tetrahydrofuran and methylene chloride which were distilled from the appropriate drying agents before use. Normal-phase column chromatography was carried out utilizing silica gel 60 (E. Merck).

Methyl 2-Amino-4-chloro-5-iodobenzoate. To a mixture of 6.84 g (27 mmol) iodine and 8.4 g (27 mmol) silver sulfate in 270 mL absolute ethanol was added 5.0 g (27 mmol) methyl 2-amino-4-chlorobenzoate (**2**). The resulting reaction mixture was stirred at room temperature for 45 min. The reaction mixture was filtered through a pad of Celite and the solvent was removed under vacuum. The residue was dissolved in 400 mL ethyl acetate and washed with saturated aqueous sodium bicarbonate (3 × 50 mL), water (3 × 50 mL) and once with brine. The organic layer was dried over magnesium sulfate, filtered and the filtrate concentrated under vacuum to afford 8.5 g (~100%) of the product as an off-white solid. ^{1}H NMR (400 MHz, $CDCl_3$): δ 3.85 (s, 3H), 5.80 (s, 2H), 6.80 (s, 1H), 8.24 (s, 1H). FAB-MS: calcd for $C_8H_7ClINO_2$ 311; found 312 (M + H, 100).

Methyl 2-*N*-Acetylamino-4-chloro-5-iodobenzoate. To a solution of 2 g (6.42 mmol) methyl 2-amino-4-chloro-5-iodobenzoate in 15 mL 1,2-dichloroethane was added 0.5 mL (7.06 mmol, 1.1 equiv) acetyl chloride. The resulting reaction mixture was heated at reflux until all solids had dissolved. The reaction mixture was cooled to room temperature and the solvent was removed under vacuum. The resulting off-white solid was triturated in 25 mL of hot methanol. The mixture was cooled in an ice bath. The solids were filtered, washed with ice-cold methanol and dried to afford 2.12 g (93%) of the product as a white solid. ^{1}H NMR (400 MHz, $CDCl_3$): δ 2.23 (s, 3H), 3.93 (s, 3H), 8.45 (s, 1H), 8.49 (s, 1H). FAB-MS: calcd for $C_{10}H_9ClINO_3$ 353; found 354 (M + H, 100).

Dimethyl 6-*N*-Acetylamino-4-chloro-1,3-isophthalate. To a solution of 2.12 g (6 mmol) methyl 2-*N*-acetylamino-4-chloro-5-iodobenzoate in 50 mL *N*,*N*-dimethylformamide and 12 mL methanol were added 1.7 mL (12 mmol, 2 equiv.) triethylamine and 0.21 g (0.30 mmol, 0.05 equiv) dichlorobis-(triphenylphosphine)palladium (II). The resulting reaction mixture was degassed and then stirred under a carbon monoxide atmosphere (1 atm) at 80 °C for 16 h. The reaction mixture was cooled to room temperature and poured into 150 mL ice/water mixture. The resulting solids were filtered, washed with ice-cold water and air-dried. Filtration of the crude product through a pad of silica gel eluting with CH_2Cl_2/EtOAc gradient (100% to 90%) gave 1.74 g (74%) of product as a white solid. ^{1}H NMR (400 MHz, $CDCl_3$): δ 2.26 (s, 3H), 3.93 (s, 3H), 3.96 (s, 3H), 8.59 (s, 1H), 8.93 (s, 1H). FAB-MS: calcd for $C_{12}H_{12}ClNO_5$ 285; found 286 (M + H, 100).

Dimethyl 6-Amino-4-chloro-1,3-isophthalate (3). To a suspension of 1.27 g (4.4 mmol) dimethyl 6-*N*-acetylamino-4-chloro-1,3-isophthalate in 15 mL methanol was added 2 mL concentrated sulfuric acid. The resulting reaction mixture was heated at reflux 1 h. The reaction mixture was cooled to room temperature and the solvent was removed under vacuum. The residue was dissolved in 150 mL ethyl acetate and washed with 10% aqueous sodium bicarbonate (3 × 75 mL), water (75 mL) and saturated aqueous sodium chloride (75 mL). The organic layer was dried over sodium sulfate, filtered and the filtrate concentrated under vacuum. The resulting off-white solid was recrystallized in methanol to afford 0.86 g (80%) of product as a white solid. ^{1}H NMR (400 MHz, $CDCl_3$): δ 3.87 (s, 3H), 3.89 (s, 3H), 6.18 (br. S, 2H), 6.72 (s, 1H), 8.54 (s, 1H). FAB-MS: calcd for $C_{10}H_{10}ClNO_4$ 243; found 244 (M + H, 100).

3,4,5-Trimethylphenylacetyl Chloride. To a solution of 9.4 g (52.7 mmol) 3,4,5-trimethylphenylacetic acid[7] in 60 mL ethylene chloride under nitrogen atmosphere at 0 °C was added via syringe 5.06 mL (58 mmol) oxalyl chloride followed by 1 drop *N*,*N*-dimethylformamide. The resulting mixture was stirred at room temperature for several hours until gas evolution ceased. Use test (reaction with methanol) of a small aliquot showed all starting material was consumed by thin-layer chromatography (hexanes/ethyl acetate, 1/1). The solvent of the reaction mixture was removed under vacuum and the resulting oil was used in the next reaction without further purification.

Dimethyl 6-*N*-(3,4,5-Trimethylphenyl)acetylamino-4-chloro-1,3-isophthalate (4). To a solution of 12.8 g (52.7 mmol) dimethyl 6-amino-4-chloro-1,3-isophthalate (**3**) in 80 mL 1,2-dichloroethane was added 10.4 g (52.7 mmol) 3,4,5-trimethylphenylacetyl chloride. The resulting reaction mixture was heated at reflux for 12 h. The reaction mixture was cooled to room temperature and the solvent was removed under vacuum. The resulting off-white solid was triturated in hot methanol. The mixture was then cooled in an ice bath, solids were filtered, washed with ice-cold methanol and dried to afford 16.6 g (78%) of product as a white solid. ^{1}H NMR (400 MHz, CD_3OD): δ 2.16 (s, 3H), 2.30 (s, 6H), 3.67 (s, 2H), 3.89 (s, 3H), 3.93 (s, 3H), 7.01 (s, 2H), 8.55 (s, 1H), 8.95 (s, 1H). FAB-MS: calcd for $C_{21}H_{22}ClNO_5$ 403; found 404 (M + H, 100).

6-Carbomethoxy-7-chloro-4-hydroxy-3-(3,4,5-trimethylphenyl)-1*H*-quinolin-2-one (5). To a solution of 2.04 g (5 mmol) dimethyl 6-*N*-(3,4,5-trimethylphenyl)acetylamino-4-chloro-1,3-isophthalate (**4**) in 20 mL dry tetrahydrofuran under nitrogen atmosphere at 0 °C was added dropwise via syringe 12.6 mL (12.6 mmol, 2.5 equiv) of a solution of sodium bis-(trimethylsilyl)amide (1.0 M in tetrahydrofuran). The resulting reaction mixture was stirred at 0 °C for 1 h then quenched with 60 mL 6 N aqueous HCl/ice (1:1). The resulting solids were stirred vigorously, filtered, washed with ice-cold water followed by ice-cold acetonitrile. The resulting off-white solid was dried in a vacuum oven at 50 °C for 16 h to afford 1.6 g (82%) of the product. ^{1}H NMR (400 MHz, DMSO-d_6): δ 2.14 (s, 3H), 2.24 (s,6H), 3.85 (s, 3H), 6.93 (s, 2H), 7.37 (s, 1H), 8.43 (s, 1H). FAB-MS: calcd for $C_{20}H_{18}ClNO_4$ 371; found 372 (M + H, 100).

4-(2-(*N*-*tert*-Butoxycarbonylazetidin-2(*S*)-yl)ethoxy)-6-carbomethoxy-7-chloro-3-(3,4,5-trimethylphenyl)-1*H*-quinolin-2-one (7). To a vigorously stirred solution of 1.35 g (6.72 mmol) *N*-*tert*-butoxycarbonylazetidine-2(*S*)-ethanol (**6**) in 67 mL dry tetrahydrofuran under nitrogen was added 2.50 g (6.72 mol) finely powdered 6-carbomethoxy-7-chloro-4-hydroxy-3-(3,4,5-trimethylphenyl)-1*H*-quinolin-2-one (**5**). To the resulting mixture was added 1.94 g (7.40 mmol) triphenylphosphine then dropwise by syringe 1.2 mL (7.4 mmol) diethyl azodicarboxylate. The resulting mixture was stirred at ambient temperature for 16 h at which time 50 mL silica gel was added to the reaction mixture. The excess solvent was removed under vacuum to provide a free flowing powder which was applied to the top of a prepacked silica gel column. The column was eluted with hexanes/ethyl acetate (65/35) to provide 3.2 g of the product contaminated with a minor amount of diethyl *N*,*N*′-hydrazinedicarboxylate. ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.37 (s, 9H), 1.71 (m, 1H), 1.83 (m,1H), 2.15 (m, 2H), 2.20 (s, 3H), 2.32 (s, 6H), 3.70 (m, 4H), 3.94 (s, 3H), 4.14 (m, 1H), 6.50 (br. S, 1H), 7.10 (s, 2H), 7.32 (s, 1H), 8.42 (s, 1H). Resonances for diethyl *N*,*N*′-hydrazinedicarboxylate: δ 1.25 (t), 4.19 (q). FAB-MS: calcd for $C_{30}H_{35}ClN_2O_6$ 554; found 555 (M + H, 100).

4-(2-(*N*-*tert*-Butoxycarbonylazetidin-2(*S*)-yl)ethoxy)-7-chloro-2-oxo-3-(3,4,5-trimethylphenyl)-1,2-dihydroquinoline-6-carboxylic Acid. To a mixture of 3.2 g (~5.8 mmol) 4-(2-(*N*-*tert*-butoxycarbonylazetidin-2(*S*)-yl)ethoxy)-6-carbomethoxy-7-chloro-3-(3,4,5-trimethylphenyl)-1*H*-quinolin-2-one (**7**) in 65 mL 95% aqueous ethanol was added 2.42 g (57.6 mmol) lithium hydroxide monohydrate. The resulting mixture was heated at 80 °C for 6.5 h. The reaction mixture was cooled to room temperature and the solvent was removed under vacuum. The residue was dissolved in a minimal amount of water then cooled to 0 °C in an ice bath. The solution was acidified to pH 2 with a saturated solution of potassium hydrogen sulfate. The aqueous solution was extracted thoroughly with ethyl acetate (3×). The resulting organic layers were combined, dried over sodium sulfate, filtered and the solvent removed under vacuum to afford 1.79 g (86%, 2 steps) of the product as a white solid. ^{1}H NMR (400 MHz, $CDCl_3$ containing 1 drop CD_3OD): δ 1.31 (s, 9H), 1.68 (m, 1H), 1.78 (m,1H), 2.12 (m, 2H), 2.25 (s, 3H), 2.36 (s, 6H), 3.64 (m, 3H), 3.70 (m, 1H), 4.12 (m, 1H), 7.01 (s, 2H), 7.27 (s, 1H), 8.40 (s, 1H). FAB-MS: calcd for $C_{29}H_{33}ClN_2O_6$ 540; found 541 (M + H), 358 (M − 182, 100).

4-(2-(*N*-*tert*-Butoxycarbonylazetidin-2(*S*)-yl)ethoxy)-7-chloro-2-oxo-3-(3,4,5-trimethylphenyl)-1,2-dihydroquinoline-6-carboxylic Acid Pyrimidin-4-ylamide (8). To a solution of 245 mg (0.45 mmol) 4-(2-(*N*-*tert*-butoxycarbonylazetidin-2(*S*)-yl)ethoxy)-7-chloro-2-oxo-3-(3,4,5-trimethylphenyl)-1,2-dihydroquinoline-6-carboxylic acid in 4 mL dry methylene chloride under nitrogen atmosphere were added 55 mg (0.45 mmol) 4-(dimethylamino)pyridine, 215 mg (2.26 mmol) 4-aminopyrimidine, and 260 mg (1.36 mmol) ethyl(dimethylaminopropyl)carbodiimide. The resulting reaction mixture was stirred at ambient temperature for 24 h, diluted with methylene chloride and transferred to a separatory funnel. The organic layer was washed with water followed by brine. The aqueous layers were back extracted with methylene chloride (3×) and the combined organic layers were dried over sodium sulfate, filtered and the solvent removed under vacuum. The residue was purified by column chromatography on silica gel eluting with ethyl acetate/hexanes (70/30 to 80/20 gradient) to provide 236 mg (84%) of the product as a white solid. ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.30 (s, 9H), 1.69 (m, 1H), 1.82 (m,1H), 2.09 (s, 3H), 2.17 (m, 1H), 2.25 (s, 6H), 3.69 (m, 3H), 3.78 (m, 1H), 4.17 (m, 1H), 7.02 (s, 2H), 7.38 (s, 1H), 8.22 (s, 1H), 8.25 (d, J = 4 Hz, 1H), 8.65 (d, J = 4 Hz, 1H), 8.90 (s, 1H), 9.47 (s, 1H). FAB-MS: calcd for $C_{33}H_{36}ClN_5O_5$ 617; found 618 (M + H, 100).

4-(2-(Azetidin-2(*S*)-yl)ethoxy)-7-chloro-2-oxo-3-(3,4,5-trimethylphenyl)-1,2-dihydroquinoline-6-carboxylic Acid Pyrimidin-4-ylamide Dihydrochloride Salt (1). To a solution of 698 mg (1.13 mmol) 4-(2-(*N*-*tert*-butoxycarbonylazetidin-2(*S*)-yl)ethoxy)-7-chloro-2-oxo-3-(3,4,5-trimethylphenyl)-1,2-dihydroquinoline-6-carboxylic acid pyrimidin-4-ylamide in 10 mL dry methylene chloride under nitrogen atmosphere was added 3 drops anisole followed by 10 mL trifluoroacetic acid. The reaction mixture was stirred at ambient temperature for 4 h at which time the volatiles were removed under vacuum. The resulting material was purified by column chromatography on silica gel eluting with methylene chloride/2 N ammonia in methanol (95/5 to 93/7 gradient) to provide 510 mg (87%) 4-(2-(azetidin-2(*S*)-yl)ethoxy)-7-chloro-2-oxo-3-(3,4,5-trimethylphenyl)-1,2-dihydroquinolin-6-carboxylic acid pyrimidin-4-ylamide. ^{1}H NMR (400 MHz, CD_3OD): δ 2.05 (m, 1H), 2.19 (m, 3H), 2.25 (s, 3H), 2.34 (s, 6H), 3.72 (m, 1H), 3.78 (m, 2H), 3.98 (m, 1H), 4.42 (m, 1H), 7.09 (s, 2H), 7.49 (s, 1H), 8.11 (s, 1H), 8.35 (d, J = 4 Hz, 1H), 8.71 (d, J = 4 Hz, 1H), 8.90 (s, 1H). FAB-MS: calcd for $C_{28}H_{28}ClN_5O_3$ 517; found 518 (M + H, 100). The free base was converted to the hydrochloride salt by dissolution in methanol and addition of a 2 N HCl solution in ether followed by removal of the solvent under vacuum to provide **1** as an off-white amorphous solid. ^{1}H NMR (400 MHz, CD_3OD): δ 2.05 (m, 1H), 2.20 (m, 3H), 2.25 (s, 3H), 2.35 (s, 6H), 3.74 (m, 1H), 3.79 (m, 2H), 3.98 (m, 1H), 4.43 (m, 1H), 7.09 (s, 2H), 7.51 (s, 1H), 8.25 (s, 1H), 8.75 (d, J = 6.8 Hz, 1H), 8.93 (d, J = 6.8 Hz, 1H), 9.25 (s, 1H). High-resolution ESI MS: calcd $C_{28}H_{28}ClN_5O_3$ MW = 518.1953; found 518.1942.

In Vitro Binding Assays.[5−8] Crude membranes prepared from rat pituitary glands or Chinese hamster ovary K1 cells stably expressing human GnRH receptors were used as the sources for GnRH receptors. 5-[^{125}I-Tyr]Buserelin (a peptidyl GnRH agonist obtained from Woods Assays) having specific activity of 1000 Ci/mmol was used as the radiolabeled ligand. Competitive binding was measured in a 50 mM Tris-HCl based buffer (pH 7.5) containing 2 mM $MgCl_2$ and 0.1% bovine serum albumin. The binding activity is reported as an IC_{50} value which is the antagonist concentration required to inhibit the specific binding of [^{125}I]buserelin to GnRH receptors by 50%.

In Vitro Functional Assay.[11] Chinese hamster ovary cells stably expressing human GnRH receptors functionally coupled to phospholipase C were used to evaluate the functional GnRH antagonism of test compounds. Clones were seeded at a concentration of 60 000 cells/mL/well in inositol-free F12 medium containing 10% dialyzed fetal bovine serum, 1% Pen/Strep, 2 mM glutamine, 500 μg/mL G418 and 1 μCi [^{3}H]inositol in 24-well tray. 48 h after seeding, cells were washed with 3 × 1 mL of PBS containing 10 mM LiCl and treated with various concentrations of test compounds for 2 h at 37 °C before addition of 0.5 nM GnRH (Sigma Chemical Co.). After incubation at 37 °C for an additional 60 min, the medium was removed and the cells were lyzed with 1 mL of 0.1 M formic acid. The trays were freeze−thawed once and the cell extract was applied to a Dowex AG1-X8 column. The column was washed with 2 × 1 mL H_2O to remove free [^{3}H]inositol and [^{3}H]inositol phosphates were eluted with 3 × 1 mL 2 M ammonium formate in 1 M formic acid. The eluate was counted in a scintillation counter. The results are reported as an IC_{50} value which is the antagonist concentration required to inhibit the GnRH-stimulated PI hydrolysis by 50%.

Intravenous Pharmacokinetics of Compound 1 in Rhesus Monkey. Compound **1** in ethanol/PEG400/saline (10/

40/50, v/v/v) was administered by the brachiocephalic vein to male rhesus monkeys (average dose, 2.64 mg/kg). Blood was collected from the femoral vein at 5, 15, 30 min and at 1, 2, 4, 6, 8, 24 h after dosing. From the blood collected at each sampling, plasma was obtained by centrifugation. Concentrations of compound **1** in plasma were determined by LC/MS/MS after liquid/liquid extraction (ethyl acetate) versus an internal standard. The limit of quantitation was 2.0 ng/mL.

The HPLC system consisted of two Shimadzu LC-600 pumps, an SCL-6B controller and an SIL-6B autoinjector. Chromatography was carried out on a Spheresorb C8 (5 μm, 4.6 × 50 mm) column using isocratic mobile phase consisting of 80% acetonitrile with 20% water with 10 mM ammonium acetate and 0.1% trifluoroacetic acid. The flow rate was 1.0 mL/min.

LC/MS/MS assays were performed on a SCIEX API III tandem mass spectrometer using the heated nebulizer interface. Mass spectra and production spectra were obtained using an ionspray interface and positive ion detection was used with argon as the collision gas.

Areas under the plasma concentration versus time curve (AUC) were determined using linear trapezoidal interpolation in the ascending slope and logarithmic trapezoidal interpolation in the descending slope. Concentrations lower than the limit of quantification were treated as zero for the purpose of calculation of AUC, AUMC, and means. The portion of the AUC from the last measurable plasma concentration to infinity was estimated by C_t/λ, where C_t represents the last measurable plasma concentration and λ is the terminal rate constant determined from the plasma concentration versus time curve by the linear regression of the elimination phase of the semilogarithmic plot. The portion of the AUMC from the last measurable plasma concentration to infinity was estimated by $t \times C_t/\lambda + C_t/\lambda^2$. Elimination half-life ($t_{1/2}$) and plasma clearance (Cl_p) were calculated by the following equations: $t_{1/2} = 0.693/\lambda$; $Cl_p = \text{dose}/AUC_{(0-\infty)}$.

In Vivo Efficacy in Rhesus Macaques. Four adult male rhesus macaques were previously administered with chronic intravenous catheters maintained by constant infusion of saline containing heparin. Catheters were tunneled sc to exit via a stainless steel tether and passed through a wall to allow for blood sampling from another room. This allowed the animals to be dosed and have blood samples collected without disturbing the animals or turning on the lights in the room. Lights were set to a 0700:20:00 h light−dark cycle. Animals were completely conditioned to this arrangement over the course of several months. They were fed on a daily schedule of 08:30 and 15:00.

All animals received compound **1** at 0.5 or 3.0 mg/kg as a single iv dose dissolved in ethanol, PEG400 and saline (10/40/50). Two hours before dosing, blood sampling began, and samples of 1 mL were collected into heparinized syringes every 20 min throughout the study. Initiation of blood sampling was at 16:00, compound or vehicle administration was at 18:00, and sampling finished at 02:00. Approximately every hour, red cells were returned to the animal via the iv catheter, using sterile technique. A total blood volume of approximately 40 mL was collected during the study. Compound **1** was dosed using a 10−12 min iv infusion. RIA was used to determine serum LH and T levels from the samples obtained at each time point. Hormone AUC was calculated using a standard graphing program for all hormone datapoints recorded during the duration of the study ($t = -130$ to 470 min).

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Supporting Information Available: Time course graphs of hormone levels (LH and T) for the other three subjects in the rhesus monkey study. This material is available free of charge via the Internet at http://pubs.acs.org.

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